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MINIREVIEW

How Innate Immune Mechanisms Contribute to Antibody-Enhanced Viral Infections▽

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Preexisting antibodies may enhance viral infections. In dengue, nonneutralizing antibodies raised by natural infection with one of four dengue viruses (DENVs) may enhance infection with a different virus by a process we term "intrinsic antibody-dependent enhancement" (iADE). In addition, nonprotective antibodies raised by formalin-inactivated respiratory syncytial virus (RSV) and measles virus vaccines have led to enhanced disease during breakthrough infections. Infections under iADE conditions not only facilitate the process of viral entry into monocytes and macrophages but also modify innate and adaptive intracellular antiviral mechanisms, suppressing type 1 interferon (IFN) production and resulting in enhanced DENV replication. The suppression observed *in vitro* has been documented in patients with severe (dengue hemorrhagic fever [DHF]) but not in patient with mild (dengue fever [DF]) secondary dengue virus infections. Important veterinary viral infections also may exhibit iADE. It is thought that use of formalin deconforms viral epitopes of RSV, resulting in poor Toll-like receptor (TLR) stimulation; suboptimal maturation of dendritic cells with reduced production of activation factors CD40, CD80, and CD86; decreased germinal center formation in lymph nodes; and the production of nonprotective antibodies. These antibodies fail to neutralize RSV, allowing replication with secondary stimulation of RSV-primed Th2 cells producing more low-avidity antibody, resulting in immune complexes deposited into affected tissue. However, when formalin-inactivated RSV was administered with a TLR agonist to mice, they were protected against wild-type virus challenge. Safe and effective vaccines against RSV/measles virus and dengue virus may benefit from a better understanding of how innate immune responses can promote production of protective antibodies.

Over the past 4 decades different lines of scientific inquiry have contributed to improved understanding of how antibody-mediated mechanisms control the severity of diseases that accompany heterotypic viral infections or that follow incomplete immunization. In the case of heterotypic infection, independent studies on the cellular and host responses to acute and chronic human and animal viral diseases provide evidence that linking of immune complexes with Fcγ receptors enhance infection severity by a mechanism we term "intrinsic antibody-dependent enhancement" (iADE) (8). Parallel studies on immunization with respiratory syncytial virus (RSV) antigens demonstrate how use of formalin-inactivated viral immunogens yields deficient Toll-like receptor (TLR) activation of B cells, defective affinity maturation, and nonprotective antibodies (14, 39). The severe wild-type viral diseases occurring in the presence of these antibodies are characterized by eosinophilia, complement fixation, and Arthus-like phenomena (7, 11, 20, 40). The research histories of these two innate immune response-triggered antibody-mediated viral immunopathologies are reviewed.

iADE. Hawkes observed enhanced plaque formation when Murray Valley encephalitis virus (MVEV) was incubated with low concentrations of antibodies during studies on neutralization using the serum-dilution, virus-constant method. A greater number of plaques were observed in chicken embryo fibroblast monolayers containing high dilutions of chicken MVEV antisera than in virus-only controls (34). In further studies it was suggested that plaque enhancement resulted from the stabilization of infectivity of virus-antibody complexes (35). Subneutralizing antibody-virus complex infection of monocytes/macrophages was subsequently described as a pathological mechanism during secondary dengue virus (DENV) infection, explaining the observation that sequential DENV infection resulted in severe disease (28, 29, 31). This led to the recognition that chicken embryo fibroblast monolayers contained 2% functional macrophages which supported MVEV infection and plaque formation in the presence of chicken MVEV antibodies (42). Importantly, this system required that the phylogenic class of donors of IgG antibodies be the same as that of donors of Fcγ-bearing cells (43).

During initial studies on ADE it had been assumed that increased virus output, which in some cases approached 100- to 1,000-fold, resulted from the avid attachment of immune complexes to FcγI and -IIa receptors (FcγRI and FcγRII, respectively), therefore yielding a larger number of cells infected in the presence than in the absence of antibodies (26, 31, 32). For example, in mouse macrophage-like cells a significant increase in attachment of West Nile virus immune complexes compared with that of naked virus particles was observed (22, 23). Using

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feline infectious peritonitis (FIP) virus (FIPV), an increased number of peritoneal macrophages were infected *in vitro* in the presence than in the absence of antibodies (54). It was also possible that immune complexes were internalized more rapidly than naked virus, as has been observed in a human immunodeficiency virus (HIV) type 1 model (64).

These mechanistic concepts of ADE changed radically as a result of studies on macrophages infected by Ross River virus (RRV) immune complexes. In humans, acute infections with RRV often evolve to a postinfection arthritis of many months' duration. When sampled, arthritis patients' synovial cells stained for RRV antigens and synovial fluids contained gamma interferon (IFN-γ). In an attempt to model this phenomenon, chronic RRV infections were established in mouse macrophage cell lines and were confirmed in primary human monocytes/macrophages (49). Remarkably, the incubation of RRV with diluted RRV antiserum resulted in enhanced infection in these cells through a complex phenomenon involving increased production of virus resulting from immune complex suppression of innate cellular immunity. The innate immune suppression involved decreasing production of reactive nitrogen radical via nitric oxide synthase 2 (NOS2) suppression and downregulation of tumor necrosis factor alpha (TNF-α) and IFN-β production through abolished interferon regulatory factor 1 (IRF-1) and nuclear factor-κB gene expression, while a marked increase in interleukin-10 (IL-10) gene transcription and protein production was observed (48, 71). Critically, this suppressive phenomenon required an infectious immune complex since the ligation of FcγR by zymosan-antibody complexes in the presence of RRV did not ablate antiviral transcription (71). Thus, rather than simply evoking an increase in the number of infected cells, RRV ADE is a complex phenomenon involving increased production of virus resulting from immune complex suppression of innate cellular immunity. The ADE phenomenon has attracted wide interest in virology because many viruses replicate in macrophages *in vivo* and manifest enhanced infections/diseases (26, 37, 72, 73).

Antibody-enhanced infections require an initial immunological event, termed "sensitization," that occurs in three settings: (i) primary infections with naturally occurring heterotypic viruses of the same genera (group 1), (ii) infection by viruses that create antigenic diversity by the rapid evolution of biologic or antigenic variants during the course of a chronic infection (group 2), and (iii) immunizations that result in incomplete protective immunity (group 3). Viruses in group 1 include the four dengue viruses, while group 2 includes lactic dehydrogenase virus, FIPV, porcine reproductive and respiratory syndrome (PRRS) virus, simian hemorrhagic fever (SHF) virus, HIV, equine infectious anemia (EIA) virus, caprine arthritis (CA) virus, and African swine fever virus (41, 82). Useful reviews are available (26, 73).

(i) Group 1: dengue viruses. In humans, severe dengue virus infections follow a stereotypical course; outcomes such as anoxia, shock, and gastrointestinal hemorrhage accompany a rapid loss of fluid from the vascular compartment due to capillary permeability occurring around the time of defervescence (12). During studies on pathogenic mechanisms, considerable effort has been directed at measuring blood cytokine levels in patients late during the acute phase of dengue illnesses, just prior to onset of shock (4). High levels of proinflammatory and immunomodulatory cytokines, including IL-10, are associated with severe disease (24). Indirect evidence suggests that cytokines mediate dengue virus vascular permeability. Infection-enhancing antibodies are a risk factor for enhanced dengue disease (44). Enhanced viremia has been shown to be an anticipatory correlate of severe disease (47, 77). Recent pathology studies of human tissues have firmly established the central role of monocytes, macrophages, and immature and mature dendritic cells as infected target cells (2, 17, 38, 81). Correlative studies have validated *in vitro* use of various human primary and continuous monocyte or macrophage cell lines as surrogate models of *in vivo* infections (5, 8, 13, 27, 30, 33, 44).

During *in vitro* ADE infection in the THP-1 cell model (THP-1 is a human monocytic Fcγ receptor-bearing continuous cell line), intracellular DENV production was increased as a result of idiosyncratic Fcγ receptor signaling (8). When immune complexes ligate FcγRI and FcγRIIA, at least two types of suppression pathways are expressed: the deoxyadenosine kinase (DAK), antigen 5 (the autophagy-related gene [Atg5-Atg12] complex), SARM (sterile alpha- and armadillo-motif-containing protein), and TANK (the TRAF family member-associated NF-κB activator) pathway plus the positive Th2 cytokine regulator IL-10 pathway (Fig. 1). Collectively, these downregulate antiviral responses in ADE-infected target cells. DAK and the Atg5-Atg12 complex of RIG-I/MDA5 (retinoid acid-inducible gene I/melanoma differentiation-associated gene 5) abolish expression of RIG-I/MDA5 and weaken the RIG-I/MDA5 signaling pathway, as monitored through levels of downstream signaling molecules: beta interferon promoter stimulator 1 (IPS-1), inducible I-κB kinase (IKKi), tumor necrosis factor receptor-associated factor 3 (TRAF-3), and TANK-binding kinase 1 (TBK-I), etc. An outcome is decreased production of type I IFN as well as interferon-activated antiviral molecules (76). Activation of SARM and TANK results in expression blockage of TLRs 3, 4, and 7 (49a). This inhibits the myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent signaling pathways, resulting in another route for type I IFN suppression. As a result of at least these two suppression pathways, ADE-infected THP-1 cells secreted reduced levels of type I IFN and at the same time suppressed the transcription and translation of IL-12, IFN-γ, and TNF-α, facilitating the expression and synthesis of the anti-inflammatory cytokines. ADE infection also suppressed the innate anti-DENV mediator, nitric oxide radicals, by disrupting the transcription of the inducible nitric oxide synthase (iNOS) gene transcription factor IRF-1 (8). This suppressive mode is believed to be mediated by IL-10 activity. IL-10 is synthesized at an early phase of ADE infection in THP-1 cells. In this experimental setting, IL-10 not only induces Th2 biasing but also operates via the suppressor of cytokine signaling (SOCS) system to suppress the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, resulting in suppression of iNOS gene expression and reduction of nitric oxide radical production. The viral enhancement effect of IL-10 is abolished with small interfering RNA specific to the IL-10 gene (76). It can be concluded that *in vitro*, iADE infection not only facilitates viral entry but also modifies innate and adaptive intracellular antiviral mechanisms, resulting in enhanced DENV replication (Fig. 1).

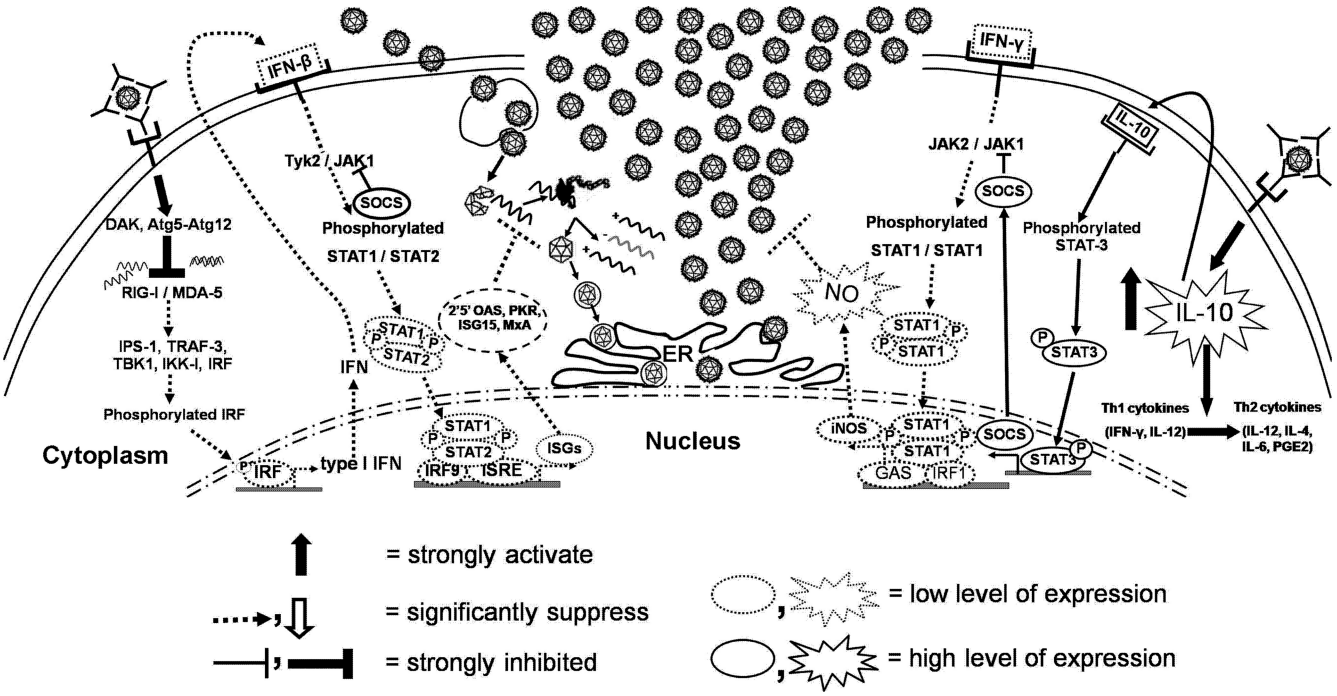

FIG. 1. Two-loop model of iADE. Ligation of FcγR by infectious DENV-subneutralizing antibody complex induces suppression of innate immune responses by (i) upregulation of negative regulators of pathogen pattern recognition, DAK, Atg5 to Atg12, SARM, and TANK, which subsequently abolish expression of RLR and TLRs and its signaling pathway, resulting in decreased type 1 interferon and proinflammatory cytokine production, which serves to suppress interferon-mediated antiviral responses, and (ii) early activation of IL-10. IL-10 potently activates the SOCS system, suppressing the JAK/STAT signaling pathway and, in turn, the interferon-signaling pathway. IL-10 is known to promote a type Th2 cytokine response, resulting in Th2-type cytokine biasing. These two loops of suppression switch off intracellular antiviral responses in DENV-infected cells under iADE conditions, resulting in the production of high numbers of infectious virions. Tyk2, tyrosine kinase 2; ISRE, interferon-stimulated response element; OAS, 2′,5′-oligoadenylate synthase; PKR, protein kinase R; ISGs, interferon-stimulated genes; GAS, gamma interferon-activated sequence; PGE2, prostaglandin E_2.

Critically, the same responses are observed *in vivo*. Genome-wide transcriptomes from peripheral blood mononuclear cells (PBMCs) collected during the acute phase from children with dengue fever (DF) or dengue hemorrhagic fever (DHF) were compared using microarray analysis (75). Patients with DHF had decreased levels of NO, reduced IFN transcripts in PBMCs, and increased blood IL-10 levels compared with patients with milder illness. IFN gene upregulation and IFN-α production were significantly elevated in patients with mild dengue illness compared with patients with severe dengue illness. In other studies, during the acute stage of severe disease, increased production of IL-10 and downregulation of multiple IFN regulatory genes were noted (10, 53, 70). The protective role of IFN in moderating dengue virus infection is clearly demonstrated in a mouse model and is suggested for humans with DF (52, 67, 68). The precise role of immune complex-elicited IL-10 production on the clinical evolution of severe dengue virus infections is not well understood but may be responsible for the observed Th1-to-Th2 shift in DHF (9). However, during ADE infection of primary monocytes, IL-10 synthesis peaked at the same serum dilution that produced peak virus yield (M. Marovich, personal communication). In addition, point mutations at the IL-10 promoter, at positions −1082 A/G, −819 C/T, and −592 C/A, result in polymorphisms that differentiate monocytes into high, intermediate, and low IL-10 producers, respectively. How these phenotypes correlate with disease severity requires more investigation.

When studied *in vitro*, the response of human myeloid cells to iADE infection differed (5a). Human monocytes, activated macrophages, and mature dendritic cells (DCs) support ADE, while immature DCs do not. Infection of macrophages by DENV type 2 (DENV2) alone or as fully neutralized immune complexes stimulated high levels of α- and β-IFN, but these were downmodulated under ADE conditions and replaced by secretion of IL-6 and TNF-α. Type I IFNs were not produced by infection with DENV2 in monocytes. More observations of human dengue illnesses are needed to better understand the iADE phenomenon. In this context, understanding the DHF in infants which accompanies primary DENV virus infections acquired during the latter half of the first year of life is important and has attracted recent research interest (46, 69). This unique clinical phenomenon not only illustrates the role played by dengue virus antibodies in modulating disease expression but also their bifurcated role: protecting after birth at high concentrations and enhancing some months later at subneutralizing concentrations. The contribution of immature DENV particles to iADE is of interest. Immature dengue virions are not infectious for human myeloid cells, but in the presence of enhancing dengue antibodies, ADE infection occurs readily (13, 65). It has been surmised that immature DENVs are

released into circulation during human infections, as antibodies to prM (immature DENV antigen) are frequently observed (13).

(ii) Groups II and III. FIP is described briefly as an example of a group II and III virus immunopathology involving ADE. Feline coronaviruses exist in two forms, The first form, feline enteric coronavirus (FECV), is a pathogen of minor significance, but a spontaneous mutation of this virus results in the second form, FIPV, which replicates in peritoneal macrophages, producing peritonitis and, occasionally, FIP, a fatal Arthus-like pyogranulomatous disease in kittens and cats, with ADE being incriminated as a disease-enhancing factor (56, 57, 80). That antibodies are pathogenic is evidenced by observations that kittens who have acquired passive maternal FIPV antibodies develop a more rapid and fulminant disease following challenge with FIPV than do seronegative kittens (80). Disease enhancement has been demonstrated in cats that were infected in the presence of vaccine-derived humoral immunity directed against the spike protein of FIPV (78). Similarly, cats immunized with a recombinant vaccinia virus expressing the spike protein of FIPV died earlier than control animals (78). In adult cats, FIPV develops during chronic infections with feline coronaviruses after FECV mutates to FIPV, gaining macrophage tropism (79). As antibody responses to FIPV are mounted, infection and disease severities are enhanced (56). In summary, antibody responses to FIPV occurring during the course of infection, passive transfer of antibodies from natural infections, and immunization with killed or recombinant vaccines have all led to enhanced FIPV disease. Enhanced disease severity has been attributed largely to the generation of nonneutralizing antibodies.

Defective activation of TLR. In humans, the severe disease syndromes that accompany reinfection following administration of killed measles virus and RSV vaccines have been recognized as antibody-dependent immunopathological phenomena distinct from the phenomenon in dengue. These viruses are responsible for a larger group of viral diseases, in which sensitization to severe outcome results from human intervention, usually by immunization using an inactivated viral antigen. On the basis of published descriptive reports, in addition to RSV and measles virus, virus diseases that have been enhanced following immunization include those caused by human metapneumovirus (hMPV), influenza A virus, rabies virus, SHF virus, FIPV, PRRS virus, HIV, EIA virus, CA virus, and Aleutian disease of mink (AD) virus (15, 26, 73). Described here are recently discovered mechanisms that control disease enhancement in RSV. It is not known which of the diseases listed above may be modified by iADE and which may be modified by defective activation of TLR, or both.

Measles and respiratory syncytial virus postvaccination infection syndromes. Formaldehyde has been widely used in the manufacture of many safe commercial vaccines, but formaldehyde-inactivated (FI) RSV (FI-RSV) and measles virus vaccines caused disastrous worsening of disease during subsequent natural infection (20, 40). This phenomenon is accompanied by a dissociation between neutralizing and glycoprotein antibody responses; enhanced IL-4, IL-5, and IL-13 responses; and tissue eosinophilia (15, 51, 59). As in the dengue model, T-cell responses in RSV infection are part of the efferent immune response contributing to tissue pathology and are identifiable once cellular infection is well established. T cells signal the host's attempts to contain and eliminate virus-infected cells. A role for T cells in RSV lung pathology following administration of killed vaccine was suspected when it was established that FI-RSV-specific antibodies, in the absence of $CD4^+$ and $CD8^+$ T cells, were not sufficient to cause disease enhancement (74). Of interest, on challenge with wild-type hMPV, cynomolgus monkeys vaccinated with formalin-inactivated hMPV, a member of the RSV family that causes a similar respiratory syndrome in humans, developed the clinical and pathological responses suggestive of enhanced immune-mediated disease (15).

Initially, one lead hypothesis was that FI-RSV pathology was an example of ADE, as in the bonnet monkey model, antibody increased virus infection of pulmonary macrophages (21, 61). However, *in vivo*, pulmonary epithelial cells and not macrophages are prime targets of infection. Also, it was observed that FI antibodies enhance RSV and measles virus disease in animal models by forming infectious immune complexes that activate complement (58, 60). This pathological response affected lung function but may also possibly reflect Th2 differentiation (1).

It had been postulated that formaldehyde-inactivated proteins raised antibodies of reduced protective capacity because of increased numbers of reactive carbonyl groups that favored Th2 immune responses following phagocytosis by macrophages via scavenger receptors (50). However, inactivation of RSV by methods other than the use of formalin also sensitized experimental animals to enhanced disease. Mice immunized with RSV that had been treated with UV radiation, purified fusion (F) protein, or a vaccinia virus RSV replicative construct experienced enhanced disease following challenge with wild-type virus (14, 55). Both high- and low-avidity antibodies were directed to the same amino acid epitope of F protein (amino acids 422 to 438). Recently, it has been shown that when tested in mice, formalin inactivation deconforms epitopes, resulting in failure of avidity maturation characterized by suboptimal maturation of dendritic cells; reduced production of activation factors CD40, CD80, and CD86; and decreased germinal center formation in lymph nodes (14). Low-avidity antibody responses to UV radiation-treated F protein could be converted to high avidity by administering antigen on five consecutive days.

Further, it was determined that TLR activation was required for avidity maturation (14). When MyD88, a downstream adaptor of most TLRs, including TLR4 and TLR7, was present, inoculation of wild-type RSV resulted in avid and protective antibody responses. In $MyD88^{-/-}$ mice, inoculation with this immunogen was not protective. Passive cellular transfer experiments using $MyD88^{-/-}$ mice revealed that TLR stimulation occurred in B lymphocytes. Of interest, when UV radiation-inactivated virus was administered along with the TLR agonists lipopolysaccharide (TLR4 agonist) and poly(I:C) plus poly(U) (TLR3 and TLR7 agonist), as opposed to the use of alum (a TLR-independent adjuvant), mice were protected against RSV challenge. In summary, poor TLR stimulation by inactivated RSV vaccines was associated with a lack of maturation and led to production of nonprotective antibodies. These antibodies were essential to the pathogenesis of enhanced disease, as they failed to neutralize wild-type RSV,

allowed unrestricted replication with secondary stimulation of RSV-primed Th2 cells. Further, low-avidity antibody contributed to disease severity through immune complex formation and deposition in affected tissue. A somewhat similar observation was made by other workers who coadministered a TLR7/8 and TLR9 agonist (CpG) with a formalin-inactivated RSV vaccine and observed a protective effect with TLR9 agonist coadministration compared with the effect in controls (39).

Discussion. This review describes how antibodies operate in the early stages of infection to direct viruses to target cells (dengue virus) or modify antigen presentation to form nonneutralizing antibodies (RSV). Critical to differences in antibody-mediated outcomes are the different principal host cells supporting infection in these two diseases. Mononuclear phagocytes are target cells for diseases initiated by ADE, while in RSV and measles virus it is likely that parenchymal lung epithelial cells support infection with limited involvement of mononuclear phagocytes. These two rather different examples of antibody-dependent viral immunopathology are unified by their interaction with the innate immune system. In both, innate immune responses of viral target or antigen-presenting cells have been modified so as to impede the potential beneficial responses that accompany and follow human infection or vaccination. The focus of this review should not be interpreted to undermine the importance of T cells in contributing to protection or pathological responses to infection during the course of elimination of virus-infected cells.

In the case of diseases in the iADE group, evidence from dengue research suggests that innate immune responses engineer increased production of virus. Presumably, disease severity is a direct correlate of infection severity. In the case of RSV, defective activation of TLR in antigen-presenting cells results in the production of nonneutralizing pathogenic antibodies. The findings described here modify the previous measles virus and RSV paradigm, ascribing atypical disease to poor antibody function due to formalin disruption of protective epitopes (50, 51, 62). Presentation of formalin-inactivated RSV or measles viral antigens results in immunogens that fail to stimulate TLR4, resulting in failure of affinity maturation (14). Nonavid, nonneutralizing, but complement-fixing antibodies raised by administration of formalin-inactivated vaccines interact to produce pathological immune complexes with wild-type viral infections that start in the respiratory tract. These antibodies were essential to the pathogenesis of enhanced disease, as they failed to neutralize RSV, permitting replication with secondary stimulation of RSV-primed Th2 cells.

Important progress has been made in identifying the role of innate immunity in successful live attenuated viral vaccines. A single injection of YF-17D induces cytotoxic T lymphocytes, a mixed Th1-Th2 profile, and neutralizing antibodies that persist for more than 30 years. These responses depend upon the fundamental role of the innate immune system, particularly TLRs and antigen-presenting cells, such as DCs. YF-17D infects DCs and signals through multiple TLRs on distinct subsets of these DCs. Computational analyses identified a gene signature including complement protein C1qB and eukaryotic translation ignition factor 2 alpha kinase 4 that correlates with $CD8^+$ T-cell responses, while a B-cell growth factor with TNFRS17 contributes to protective neutralizing antibody responses (63).

It is important to note that for many viral diseases, formalin-inactivated antigens have produced highly efficacious vaccines. Japanese encephalitis and poliomyelitis are examples (16, 19, 36). The mechanism of protection against influenza by formalin-inactivated antigens is less clear (3, 6, 18, 45, 66). The work cited suggests that safe and effective RSV vaccines for infants may be feasible by administering vaccines with TLR agonists that raise neutralizing antibodies which achieve protective efficacy similar to those elicited by live virus inoculation. This may have special resonance to developers of tetravalent dengue vaccines. Deriving live attenuated vaccines that provide tetravalent immunity following a single dose has proved to be difficult (25). The role of iADE in the large group of predominantly veterinary macrophage-tropic viral pathogens in groups II and III is yet to be firmly established. Use of vaccines composed of carefully characterized inactivated or subunit antigens with selective adjuvants may result in a more balanced and protective immune response. Answers may lie with a better understanding and use of innate immunity.

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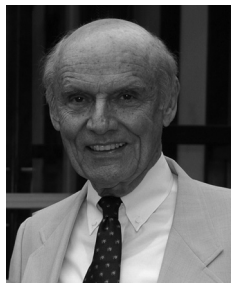

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